Lateralization of short- and long-term visual memories in an insect

Article (Accepted Version)

Lateralisation of short- and long-term visual memories in an insect

A. Sofia David Fernandes[1,2,3], Jeremy E. Niven[1,3]

[1] School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK.

[2] School of Engineering & Informatics, University of Sussex, Falmer, Brighton BN1 9QJ, UK.

[3] Centre for Computational Neuroscience & Robotics, University of Sussex, Falmer, Brighton BN1 9QG, UK.

Abstract

The formation of memories within the vertebrate brain is lateralised between hemispheres across multiple modalities, however, in invertebrates evidence for lateralisation is restricted to olfactory memories, primarily from social bees. Here we use a classical conditioning paradigm with a visual conditioned stimulus to show that visual memories are lateralised in the wood ant, *Formica rufa*. We show that a brief contact between a sugar reward and either the right or left antenna (reinforcement) is sufficient to produce a lateralised memory, even though the visual cue is visible to both eyes throughout training and testing. Reinforcement given to the right antenna induced short-term memories whereas reinforcement given to the left antenna induced long-term memories. Thus, short- and long-term visual memories are lateralised in wood ants. This extends the modalities across which memories are lateralised in insects and suggests that such memory lateralisation may have evolved multiple times, possibly linked to the evolution of eusociality in the Hymenoptera.

1. Introduction

Brain regions and the neural circuits they contain can show considerable lateralisation, even in the brains of bilaterally symmetric animals that show little asymmetry in their gross morphology. Such lateralisation appears to be important in the formation of memories in humans across many sensory modalities (for review see Rogers, 2014). For example, cortical areas of the left hemisphere are more likely to be involved in storing memories of verbal information, whereas right cortical areas are more engaged in storing visual information (Gazzaniga, 2000). Spatial memories in humans are also lateralised, being transferred from the right hippocampus to the right side of the prefrontal cortex (reviewed in Burgess *et al*., 2002). Lateralisation of memory formation is not restricted to humans, however, having been extensively demonstrated in chicks (Moorman and Nicol, 2015), and with some evidence of lateralisation in both zebra fish (Rogers and Andrew, 2002) and rodents (Jordan and Pytte, 2017).

Whether the lateralisation of memory formation across different modalities is a common feature of brains and nervous systems remains unclear. Few studies have demonstrated functional lateralisation associated with memory formation within the numerous phyla commonly termed the invertebrates (Frasnelli, 2013). Apart from a study on the lateralisation of olfactory memory formation in the

procerebrum of the slug, *Limax* (Matsuo *et al*., 2010), most evidence comes from the insects. Nevertheless, the majority of these studies have focussed upon a single modality and type of learning, appetitive classically conditioned olfactory memories in bees (Letzkus *et al*., 2006; Rogers & Vallortigara, 2008; Anfora *et al*., 2010; Frasnelli *et al.*, 2011; Rigosi *et al*., 2011; Haase *et al*., 2011; Guo *et al.*, 2016), though one study has found lateralisation of aversive olfactory memories in groups of fruit flies (Pascual *et al*., 2004).

The association between lateralisation and olfactory memory has been studied in both solitary mason bees (Anfora *et al*., 2010) and social bees from three tribes within the Apinae, namely honeybees (Letzkus *et al*., 2006; Rogers & Vallortigara, 2008), stingless bees (Frasnelli *et al.*, 2011), and bumble bees (Anfora *et al*., 2011). Lateralisation is evident in the formation of short-term olfactory memories in social bees, which form stronger memories following training with the right antenna. Conversely, long-term memories in social bees are lateralised so that stronger memories are formed in response to inputs from the left antenna (Letzkus *et al*., 2006; Rogers & Vallortigara, 2008; Frasnelli *et al.*, 2011; Anfora *et al*., 2011).

To determine whether such lateralisation of short and long-term memory formation in insects occurs within sensory modalities other than olfaction, we assessed whether visual memories are lateralised. To our knowledge, no studies have investigated the lateralisation of visual memories in insects, despite their importance to numerous aspects of insect behaviour (e.g. Collett and Collett, 2002; Collet *et* al., 2013; Collett and Zeil, 2018), though honeybees can learn an association with a visual cue more effectively with only the right eye than with only the left (Letzkus *et al.*, 2007). We used wood ants because they are a model system for studying visual navigation (reviewed in Buehlmann *et al*., 2016) and have been shown to form visual memories in a classical conditioning paradigm (Fernandes *et al*., 2018). Using this paradigm, we show that short- and long-term visual memories are lateralised in wood ants. To our knowledge, this is the first demonstration of lateralised visual memory formation in an insect, with broad implications for our understanding of visual memories in insects and the evolution of lateralisation in memory formation.

2. Material and Methods

(a) Animals and preparation

Red wood ant workers (*Formica rufa* L.) from three different colonies were used for the experiments. These colonies were collected from Ashdown Forest, Sussex, UK (N 51 4.680, E 0 1.800) in June 2017 and June and August 2018. They were maintained indoors for at least 2 months prior to the experiments commencing, during which they were kept at 26°C, under a 12 h light:12 h dark cycle and fed with sucrose (333 g/L). Prior to training, colonies were starved for at least 2 days to increase ants' motivation to feed. Ants were selected from the colony on the day of training and harnessed to a custom-made holder (see Fernandes *et al.*, 2018).

(b) Training

Ants were trained to associate a visual cue with a sucrose reward as described in Fernandes *et al.* (2018). We performed six different types of training to investigate the role of the antennae during visual associative learning. Paired Right (PR) and Paired Left (PL) ants experienced right or left antenna contact by the US prior to touching with the mouthparts, respectively. Paired Bilateral (PB) ants were trained with US touching both antennae prior to feeding, and data were pooled with that from Fernandes *et al*. (2018). No touch to the antennae (NTA) ants had their antenna attached to an insect pin with low melting temperature wax to prevent them contacting the reward (US) during training.

Unpaired controls were carried out as described in Fernandes *et al.* (2018). Unpaired Right (UPR) and Unpaired Left (UPL) ants experienced right or left antenna contact by the US prior to contact with the mouthparts, respectively. An additional unpaired group, Randomized Unpaired Left (RUPL), also experienced training identical to that of the UPL cohort except the CS and US were presented in a randomized order. A CS-only (CSO) control group had the CS (plus syringe) presentation for the same number of trials, but without the US contact to antennae or mouthparts. A US-only (USO) control was given paired training but with the CS removed and replaced with a white circular cue (10 cm diameter) to remove all potential visual cues generated during CS delivery except the syringe tip and sucrose drop. The USO did not differ in memory formation from the CSO (supplementary table 1) and is not further discussed here. For all types of training, half of the ants were trained with their right side facing the experimenter and the other half with the left, to account for potential environmental asymmetries that could be learnt during training. In both cases, the visual stimulus faced the ant and was seen by both eyes.

(c) Testing

We tested all groups in the same set-up with a 10 second CS presentation either 10 minutes, 1 hour or 24 hours after training. Ants tested at 10 minutes were not tested at any other time points, but all ants tested at 1 hour were also tested at 24 hours. After testing, ants were briefly presented with the sugar reward to assess whether they were still motivated to feed. Ants that did not feed after testing were excluded from analysis. All ants were kept harnessed until the last test ended. Ants harnessed overnight for 24 hour testing were kept in a dark and humid environment.

(d) Scoring and statistical analysis

We scored whether ants performed the maxilla-labium extension response (MaLER) during the 10 second presentation of the conditioned stimulus (CS) for each training trial and test (see Fernandes *et al*., 2018). All statistical analyses were performed using R (RStudio v1.0.143). To analyse whether the propensity to perform MaLER increased during training and whether it differed among different

training groups, we ran a logistic regression model with mixed effects (Bates, 2010), using the 'lme4' package. Models were implemented using the `glmer` command incorporating fixed (trial number, training type) and random (individual) effects (see supplementary materials). We used Barnard's test (Barnard, 1989) to compare the proportion of responses in each group during each test with the 'barnard' package. One-tailed or two-tailed Barnard's tests were applied depending on the specific test (see supplementary materials). To determine whether ants responding during testing were also those that learnt, we used a classification of 'learners' or 'non-learners' established in Fernandes *et al.* (2018); those individuals that responded on 4 or more of the 10 training trials were 'learners'. Bonferroni corrections (Bonferroni, 1936) were applied when appropriate (see supplementary materials).

3. Results

(a) Unilateral antennal reinforcement is sufficient for ants to learn the association between a visual cue (CS) and a sugar reward (US)

During each paired training trial, ants were presented with the CS (visual cue) for 10 seconds, followed by a brief contact between the US (sucrose reward) and one antenna (supplementary figure 1a, b), which we hereafter refer to as reinforcement. The ant was then allowed to feed from the US for 5 seconds, during which both antennae made contact with the US (supplementary figure 1c). Each training bout was separated from the next by a five minute interval. The majority of ants did not perform the Maxilla Labium Extension Reflex (MaLER) when presented with the visual cue on the first trial but during training their performance increased until ~30 to 40% for ants that were trained with reinforcement given to the right antenna (PR; figure 1a). The same training regime but with reinforcement given to the left antenna led to ~20 to 30% of ants performing MaLER (PL; figure 1a). We used a logistic regression to compare the probability of ants performing MaLER in the PR and PL training regimes (see Material and Methods). There was no significant difference in the percentage of ants performing MaLER in the PR and PL training regimes (Logistic regression, $N=180$, $z=1.26$, $p=0.21$), and the performance of MaLER in response to presentation of the CS increased significantly during both PR and PL training (Logistic regression; PR: $N=88$, $z=6.17$, $p<0.01$; PL: $N=92$, $z=3.48$, $p<0.01$). Furthermore, the direction that ants faced during training did not influence learning (Logistic regression, $N=180$, $z=0.32$, $p=0.75$; see supplementary materials).

To ensure that ants learnt the CS-US association, we performed unpaired training in which the CS and US were presented alternatively with a two and a half minute interval. The percentage of ants performing MaLER when subjected to unpaired training with reinforcement given to the right (UPR) or left antenna (UPL) increased during training (Logistic regression, UPR: $N=83$, $z=3.37$, $p<0.01$; UPL: $N=89$, $z=3.8$, $p<0.01$), but to a lesser extent than during paired training (figure 1b). Indeed, the percentage of ants performing MaLER was significantly lower during unpaired compared to the equivalent paired training (Logistic regression; PR-UPR: $N=171$, $z=-3.35$, $p<0.01$; PL-UPL: $N=181$,

z=-2.68, p<0.01). We performed a further control in which the CS was presented without the US, a CS-only control (CSO; figure 1b). CSO ants did not significantly increase in their performance of MaLER during training (Logistic regression, N=82, z=1.92, p=0.054). PR and PL ants also showed significantly higher percentage of MaLER than did CSO control ants (Logistic regression, N=262, PR-CSO: z=5.42, p<0.01; PL-CSO: z=4.33, p<0.01).

To determine whether unilateral antennal reinforcement with the US produced differences in learning compared to bilateral antennal reinforcement, we compared PR and PL trained ants with bilaterally trained counterparts (PB ants). Both PR and PL ants performed MaLER in response to the visual cue significantly less during training than did PB ants (Logistic regression; PR-PB: N=263, z=-2.29, p=0.02; PL-PB: N=263, z=-3.54, p<0.01; figure 1a). Thus, paired training with reinforcement given to a single antenna is sufficient for ants to form an association between the visual cue (CS) and the sugar reward (US), but reinforcement to both antennae during training results in more ants learning the association.

(b) Right antennal reinforcement is necessary for short-term visual memory formation

We tested whether ants retained a short-term memory after the end of each of the training regimes. Ten minutes after training, more than 50% of the PB and PR ants performed MaLER when presented with the CS (figure 2a). In comparison, ten minutes after the UPR, PL, UPL and CSO regimes only 10-20% of ants performed MaLER (figure 2a). The proportion of ants performing MaLER following PR training was significantly higher than those ants subjected to PL training (Barnard's test, PR-PL: N=74, df=72, p<0.01). The proportion of ants performing MaLER following PR training was also significantly higher than ants subjected to UPR training (Barnard's test; PR-UPR: N=73, df=72, p<0.01). In contrast, the proportion of ants performing MaLER following PL training did not differ significantly from the proportion of ants subjected to UPL training (Barnard's test, N=75, df=74, p=0.18). Thus, the paired presentation of CS and US coupled with right antennal reinforcement is necessary and sufficient for the formation of short-term memory.

A pattern similar to that observed at 10 minutes was also evident one hour after training (figure 2b). Ants subjected to the PR training performed MaLER significantly more than the PL (Barnard's test, N=106, df=104, p=0.03) and the UPR ants (Barnard's test, N=98, df=97, p=0.03), but PL ants did not perform MaLER more than the UPL ants (Barnard's test, N=106, df=105, p=0.28). Moreover, the proportion of PR ants that responded to the CS at 10 minutes or 1 hour after training was significantly higher for ants classified as 'learners' during training (see Material and Methods) than those that were not (Barnard's test; 10 min: N=37, df=36, p=0.036; 1h: N=51, df=50, p=0.003). Thus, paired CS-US presentation coupled with right antennal reinforcement is necessary and sufficient for the formation of short-term memory at both 10 minutes and 1 hour.

Furthermore, PR ants did not differ in the extent to which they performed MaLER in comparison to PB ants at 10 minutes or 1 hour (Barnard's test, 10 min: N=52, df=50, p=0.96; 1h: N=66,

df=64, p=0.98). Indeed, PB and PR trained ants were the only ones performing MaLER significantly more than the CSO in both tests (supplementary table 1). This suggests that although the percentage of responses to CS presentation during training was significantly lower for PR ants than PB ants, unilateral right antennal contact is sufficient for short-term memory formation and recall.

(c) Left antennal reinforcement is sufficient for long-term visual memory formation

To detect a long-term memory, we tested ants from each of the training regimes 24 hours after training was completed. In contrast to the short-term memory tests, less than 10% of ants subjected to PR training responded to the CS at 24 hours. Indeed, the percentage of ants performing MaLER in response to CS presentation 24 hours after PR training did not differ significantly from that of the cohort of ants subjected to UPR training (Barnard's test, N=78, df=77, p=0.37). Of the 39 PR ants that were tested at both 1 and 24 hours, 33% responded to the CS presentation by performing MaLER on the first test (1 hour) but not the second (24 hours), while only 0.5% responded at 24 but not 1 hour and just 0.2% of the ants responded in both tests. There was a significant reduction in the performance of MaLER in response to CS presentation from 1 to 24 hours after training for the PR ants (Barnard's test, N=39, df=38, p<0.01).

Conversely, around 30% of those ants subjected to PL training performed MaLER upon CS presentation (figure 2c). The percentage of PL trained ants performing MaLER was significantly higher than those ants subjected to PR training (Barnard's test, N=80, df=78, p<0.01). Of those PL ants that were tested both at 1 and 24 hours, 28% performed MaLER on the second test (24 hours) but not the first (1 hour), while only 1% responded to the CS at 1 hour but not at 24 hours and 0.5% in both tests. There was a significant increase in the performance of MaLER in response to CS presentation between 1 and 24 hours (Barnard's test, N=40, df=39, p<0.05). Furthermore, the proportion of ants that responded to the CS at 24 hours was significantly larger for ants that were classified as 'learners' during training (see Material and Methods) than ants that were not (Barnard's test, N=40, df=39, p=0.014).

The percentage of UPL ants that performed MaLER upon presentation of the CS at 24 hours was indistinguishable from PL ants (Barnard's test, N=81, df=80, p=0.27). This suggests that the formation of long-term memory produced by PL training is less sensitive to the CS-US separation than is short-term memory produced by PR training. To test this, we exposed ants to unpaired randomised training (RUPL) in which the order of CS and US presentation is randomised. RUPL ants showed greater variability during training without a clear trend for an increase of MaLER performances with training (Logistic regression, N=44, z=0.079, p=0.94; supplementary figure 2a). The RUPL ants were indistinguishable from UPL ants in their performance of MaLER in response to the CS presentation during testing 1 hour (Barnard's test, N=96, df=95, p=0.29; supplementary figure 2b). At 24 hours, 20% of RUPL trained ants performed MaLER, which was not significantly different than PL and UPL trained ants (Barnard's test; RUPL-PL: N=81, df=80, p=0.03; RUPL-UPL: N=82, df=81, p=0.29; supplementary figure 2c). However, only the PL group performed MaLER significantly more than the

CSO at 24h (supplementary table 1). Taken together, left antennal reinforcement is necessary and sufficient for the formation of long-term memories, although they tend to be less specific than short-term memories.

(d) Antennal reinforcement is necessary for visual learning and memory formation

To determine whether antennal reinforcement is necessary for the formation and retention of visual memories, an additional group of ants was trained to associate the visual CS with the sucrose reward given to the mouthparts without touching the antennae (not touching antennae, NTA). Spontaneous responses from these ants were higher than in other training groups, approximately 10% on the first trial (figure 1a), but MaLER performance did not increase during training (Logistic regression, N=59, $z=1.34$, $p=0.18$). Furthermore, NTA ants did not respond significantly more than the CSO in any of the tests performed (figure 2; supplementary table 1). Thus, antennal contact with the sugar reward is necessary for learning and forming short- and long-term memories of the visual CS.

4. Discussion

We investigated whether visual memories are lateralised within an insect brain by using an associative learning paradigm applied to workers of the red wood ant, *Formica rufa*. We found that visual memories are indeed lateralised; a brief contact with the right antenna (reinforcement) produces a short-term memory present 10 minutes after the end of training and persisting for at least one hour (figure 2a,b). We also showed that wood ants can form lateralised long-term, classically conditioned visual memories; left antennal reinforcement produces a long-term memory that is present at 24 hours despite the absence of memory at 10 minutes or one hour after training (figure 2b). The strength of the memory formed by unilateral antennal contact was indistinguishable from that formed by bilateral antennal contact at 10 minutes, 1 hour and 24 hours after training, suggesting that bilateral memory may be composed of separate unilateral short- and long-term memories. The lateralisation of memory produced by unilateral antennal reinforcement arises even though there are no differences in learning, and despite both antennae contacting the sucrose reward (US) whilst the ant was feeding.

(a) Relevance and quality of our measures

It is important to consider the biological relevance and reliability of our training and testing paradigms. Studies of lateralisation must be careful to ensure the absence of environmental asymmetries that could influence results. We excluded visual asymmetries by surrounding ants with a white arena for training and testing, ensuring any remaining visual asymmetries did not influence the lateralisation by placing ants so that either their right or left eye faced the experimenter during each training condition. Ants were harnessed in the same way in both conditions, again ensuring consistency and avoiding introducing asymmetries from additional sources.

We evoked associative visual memories in wood ant workers using an appetitive classical conditioning paradigm (Pavlov, 1897). Such paradigms have been widely used to evoke associative memories in insects (*e.g.* Bitterman *et al*., 1983; Guerrieri and d'Ettorre, 2010; Simões *et al*., 2011), coupling a reward (US) with a stimulus (CS) that later becomes sufficient to evoke a response alone. Our training paradigm did not test the specific feature that ants learnt but instead focussed on associative memory formation, distinguishing this from non-associative memory processes through a series of controls to ensure that ants responded only to the pairing of US with visual CS. Ants tested at 24 hours had already been tested at 1 hour. This raises the possibility that the 1 hour test may extinguish memories formed during training. However, there is no indication that the memory of paired bilaterally (PB) trained ants is extinguished by the 1 hour test. Moreover, extinction can neither account for memory lateralisation because the all ants were subjected to this test nor explain the presence of a 24 hour memory in paired left antenna trained (PL) ants despite no prior short-term memory. This suggests that the lateralisation of short- and long-term memory we observed is reliable.

The unconditioned response (UR) was the maxilla-labium extension response (MaLER). Although originally described for olfactory learning (Guerrieri and d'Ettore, 2010; Guerrieri *et al*., 2011), MaLER has been used before to assess visual learning and memory (Fernandes *et al*. 2018). The response to the visual CS was initially low but increased when paired with an appetitive US, sucrose, to ~40% of the ants responding per trial. The visual cue was visible to both eyes and lateralisation was produced through right or left antennal reinforcement, emphasising the robustness of this lateralisation. Despite differences in methodology, our training paradigm yielded similar learning rates (40%) to previous studies with honeybees using classical conditioning of a visual cue (Hori *et al*., 2007; Balamurali *et al*., 2015; Niggebrügge *et al*., 2009). We did not ablate or cover the antennae during training or testing, though paradigms for visual classical conditioning in honeybees have involved removing the antennae ostensibly to improve learning rates (Hori *et al.,* 2007; Niggebrügge *et al*., 2009; but see Balamurali *et al*., 2015). To our knowledge, only one study has tested memory recall at long time periods in honeybees with 30% responding to the visual cue after 24 hours (Niggebrügge *et al.,* 2009), a similar percentage to our observations for ants trained with reinforcement given to both antennae or only the left antenna.

To our knowledge only one other study on honeybee workers directly addresses the lateralisation of visual associations (Letzkus *et al*., 2007). Comparisons between the present study and are difficult because Letzkus *et al*. (2007) investigated associative learning using unilateral presentation of the visual stimulus (CS) producing a marked difference in learning between left and right eye presentations. In contrast, our training did not produce a difference in learning between PR and PL cohorts. Moreover, Letzkus *et al*. (2007) did not investigate the lateralisation of memory. Thus, to our knowledge, this is the first study to demonstrate the lateralisation of short- and long-term associative visual memories in insects.

(b) A model of visual memory lateralisation in wood ants

Visual memory lateralisation in wood ants is revealed only through unilateral antennal reinforcement. Consequently, we do not expect to observe deficits in visual memory formation during behaviours in which both antennae are used. Indeed, only if antennal use was strongly lateralised during behaviours such as feeding, so that contact was exclusively between one antenna and the food prior to ingestion, would we expect memory lateralisation be revealed. Where both antennae contact the food, we would expect both short- and long-term memory formation as occurs in the bilateral paired trained ants. Antennal movements during feeding on aphid honeydew are unknown but wood ants' antennal movements are biased during trophallaxis (Frasnelli *et al.*, 2012). Even so, both antennae are used during trophallaxis suggesting that memory formation is unlikely to be lateralised. Bilateral antennal reinforcement produces short- and long-term memories of equivalent strength to unilateral training with the right or left antenna, respectively. The most parsimonious explanation is that short-term memories are formed on the right subsequently disappearing whilst long-term memories are formed on the left without prior formation of short-term memories. Thus, short- and long-term memories may be formed in parallel on either side of the brain during behaviours in which both antennae contact the reward equally.

This interpretation is supported by several additional lines of evidence. Firstly, ants that received paired training without antennal contact to the reward at any time (NTA) do not form memories of the association. This demonstrates that that antennal contact with the reward is necessary for visual memory formation. Secondly, memory transfer between the right and left sides of the brain, which has been reported for olfactory memories in honeybees (Sandoz & Menzel, 2001; Malun *et al.*, 2002a; Sandoz *et al*, 2003; Komischke *et al.*, 2005), is unlikely to account for visual memory lateralisation in wood ants. During testing, the visual stimulus (CS) was presented to both eyes simultaneously. Were there any transfer of memory, we would expect that the short-term memory formed by PR trained ants would be transferred allowing these ants to respond to the visual stimulus (CS) at 24 hours. Thirdly, differences exist in the specificity of short- and long-term memories. Specifically, a high proportion of UPL ants showed a 24 hour memory after training, though UPR ants showed no memory at 10 minutes and 1 hour. This implies that short-term memories are more sensitive to the temporal separation of the CS and US during training than long-term memories, and that long-term memories are not merely short-term memories that have been consolidated.

(c) Functional relevance of memory lateralisation

Visual memory lateralisation is likely to have functional implications at the level of the neural circuits encoding the memory. Lateralisation may permit the different cellular mechanisms responsible for the coding of short-term versus long-term memories to occur in parallel. This may be advantageous because it would avoid pronounced gaps in memory that occur in the transitions between these different memory

types (Marra *et al*., 2013). The separation of short- and long-term memories on different sides of the brain has been suggested to permit the acquisition of new memories without interference from long-term memories (Frasnelli *et al*., 2010). Although the model Frasnelli *et al*. (2010) proposed was for olfactory memories in bees, aspects can be applied to visual memories in ants. It is possible that by retaining long-term memories on only one side of the brain, new short-term memories may be acquired by the opposite side. In such cases, these new short-term memories may allow ants to sample new food sources or novel locations. Such a direct role is not the only functional advantage that memory lateralisation may convey. Lateralisation may be advantageous because it avoids forming and storing a memory of the same association in parallel in the two hemispheres of the brain. Although such redundancy may ensure that memories are less prone to becoming corrupted because a second copy exists, this redundancy incurs costs in terms of energy consumption and in the total capacity for storage of additional memories. Lateralisation would reduce redundancy, saving energy and space within neural circuits (for review see Niven and Laughlin, 2008; Burns *et al*., 2010). Saving energy and memory storage capacity may be particularly important within relatively small brains such as those of ants with small numbers of neurons and low energy availability (Niven and Farris, 2012).

(d) Implications for memory lateralisation in the insects

The wood ant workers within our study were taken from multiple colonies within the same region raising the possibility that they could be genetically related, and implying population-level rather than individual-level lateralisation of visual memory. Both individual- and population-level lateralisation are well-documented within the insects. In species such as desert locusts (Bell and Niven, 2014, 2016), fruit flies (Buchanan *et al*., 2015), and antlions (Miler *at al*., 2017) behaviours are lateralised among individuals (individual-level lateralisation). Conversely, cockroaches (Cooper *et al*., 2011) and giant water bugs (Kight *et al*., 2008) show a consistent bias among most individuals within a population (population-level lateralisation). However, the majority of evidence for population-level lateralisation comes from eusocial insects such as leaf cutting ants (Jasmin and Devaux, 2015), bumble bees (Kells and Goulson, 2001) and honeybees (Rogers & Vallortigara, 2019).

Recent studies have shown that individual-level behavioural lateralisation occurs even in eusocial species and that, conversely, population-level lateralisation is observed during interactions between individuals of solitary species (Kamimura, 2006; Frasnelli *et al*., 2012; Lang and Orgogozo, 2012; Kamimura *et al*., 2014; Rogers *et al*., 2013; Romano *et al*., 2016a,b; Benelli *et al*., 2017a,b). Bell and Niven (2018) have argued that whether a trait is lateralised at the individual- or population-level depends upon the specific selective pressures to which the behaviour is subjected. Consequently, if different selective pressures act on distinct behaviours, it is possible for an individual to show individual- or population-level lateralisation in distinct behaviours. Thus, social behaviours that benefit from coordinated actions are lateralised at the population-level in several insect species. This coordination of lateralisation in the same direction across most of the individuals within a population

could drive the lateralisation of neural circuitry, depending on the strength of sociality within the species. In turn, this could drive non-social behaviours to be equally lateralised, even when they do not confer a specific advantage to the individual or group of individuals though evidence for this is lacking. In eusocial insect species, the low levels of competition between individuals likely promotes population-level lateralisation of social and non-social behaviours alike.

Whether the population-level, as opposed to individual-level, lateralisation of visual memories in ants confers specific advantages remains unclear. The population-level lateralisation of short- and long-term of visual memory formation in wood ants shows similarities to that of olfactory memories in honeybees and bumble bees (Letzkus *et al*., 2006; Anfora *et al.*, 2010; Anfora *et al.*, 2011). Yet not all bees show similar patterns of lateralisation; solitary mason bees (*Osmia bicornis*) do not show olfactory memory lateralisation (Anfora *et al*., 2010). One explanation for this pattern is that honeybees, bumble bees and stingless bees are eusocial whereas mason bees are solitary; the evolution of population-level lateralisation of olfactory memories in bees being due to the specific advantages of lateralisation conferred by eusocial environments in which conflict between individual workers is low (Anfora *et al*., 2010; for review see Niven and Frasnelli, 2018).

Previous studies of memory lateralisation in insects have focussed primarily on olfactory memories, though one study, Letzkus *et al*. (2007), investigated the lateralisation of visual learning. By demonstrating the lateralisation of visual memories, we show that memory lateralisation is not restricted to a specific modality. Furthermore, wood ants are eusocial like honeybees, bumble bees and stingless bees but the lineage of ants is an independent evolution of eusociality within the order Hymenoptera (Peters *et al*, 2017). Thus, the presence of visual memory lateralisation in wood ants is also, to our knowledge, the first evidence of memory lateralisation outside one family of bees (Apidae). Because of the absence of information about lateralisation of memory formation in more basal lineages within the Hymenoptera, we cannot be certain whether memory lateralisation has evolved independently in ants and social bees or is present in more basal lineages but has been lost in solitary bees.

Acknowledgements We thank Justine Crevel for assistance with experiments, and Paul Graham, Tom Collett, Cornelia Buehlmann and Chris Buckley for valuable discussions and for reading and/or commenting on earlier versions of the paper.

Competing interests The authors declare no competing or financial interests.

Author contributions A.S.D.F. and J.E.N. conceived the project, designed and performed the experiments and analysis and wrote the paper. Funding Financial support came from a Sussex Neuroscience 4-year programme studentship (A.S.D.F.) and the BBSRC BB/ R005036/1 (J.E.N.).

References

Anfora, G., Frasnelli, E., Maccagnani, B., Rogers, L.J. and Vallortigara, G. (2010). Behavioural and electrophysiological lateralization in a social (*Apis mellifera*) but not in a non-social (*Osmia cornuta*) species of bee. *Behavioural brain research*, **206**, 236-239.

Anfora, G., Rigosi, E., Frasnelli, E., Ruga, V., Trona, F., & Vallortigara, G. (2011). Lateralization in the invertebrate brain: left-right asymmetry of olfaction in bumble bee, *Bombus terrestris*. *PLoS One*, **6**, e18903.

Balamurali, G. S., Somanathan, H. and De Ibarra, N. H. (2015). Motion cues improve the performance of harnessed bees in a colour learning task. *Journal of Comparative Physiology A,* **201**, 505-511.

Barnard, G. A. (1989). On alleged gains in power from lower p-values. *Statistics in Medicine*, **8**, 1469-1477.

Bates, D. M. (2010). *lme4: Mixed-Effects Modeling with R*. New York: Springer

Bell, A. T., & Niven, J. E. (2014). Individual-level, context-dependent handedness in the desert locust. *Current Biology*, **24**, R382-R383.

Bell, A.T., Niven, J.E. (2016). Strength of forelimb lateralization predicts motor errors in an insect. *Biol. Lett.* **12**, 20160547. doi: 10.1098/rsbl.2016.0547.

Benelli, G., Romano, D., Kavallieratos, N., Conte, G., Stefanini, C., Mele, M., Athanassiou, C. and Canale, A. & Canale, A. (2017). Multiple behavioural asymmetries impact male mating success in the khapra beetle, *Trogoderma granarium. Journal of Pest Science*, **90**, 901-909.

Benelli, G., Romano, D., Stefanini, C., Kavallieratos, N. G., Athanassiou, C. G., & Canale, A. (2017). Asymmetry of mating behaviour affects copulation success in two stored-product beetles. *Journal of pest science*, **90**, 547-556.

Bitterman, M. E., Menzel, R., Fietz, A., & Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *Journal of comparative psychology*, **97**, 107-119.

Bonferroni, C. (1936). Teoria statistica delle classi e calcolo delle probabilita. *Pubblicazioni del R Istituto Superiore di Scienze Economiche e Commericiali di Firenze*, **8**, 3-62.

Buchanan, S. M., Kain, J. S., & De Bivort, B. L. (2015). Neuronal control of locomotor handedness in *Drosophila. Proceedings of the National Academy of Sciences USA*, **112**, 6700-6705.

Buehlmann, C., Woodgate, J. L., & Collett, T. S. (2016). On the encoding of panoramic visual scenes in navigating wood ants. *Current Biology*, **26**, 2022-2027.

Burgess, N., Maguire, E. A., & O'Keefe, J. (2002). The human hippocampus and spatial and episodic memory. *Neuron*, **35**, 625-641.

Burns, J. G., Foucaud, J., & Mery, F. (2010). Costs of memory: lessons from 'mini' brains. *Proceedings of the Royal Society B: Biological Sciences*, **278**, 923-929.

Collett, T. S., & Collett, M. (2002). Memory use in insect visual navigation. *Nature Reviews Neuroscience*, **3**, 542.

Collett, M., Chittka, L., & Collett, T. S. (2013). Spatial memory in insect navigation. *Current Biology*, **23**, R789-R800.

Collett, T. S., & Zeil, J. (2018). Insect learning flights and walks. *Current Biology*, **28**, R984-R988.

Cooper, R., Nudo, N., González, J. M., Vinson, S. B., & Liang, H. (2011). Side-dominance of *Periplaneta americana* persists through antenna amputation. *Journal of insect behavior*, **24**, 175-185.

Fernandes, A.S.D., Buckley, C.L. and Niven, J.E. (2018). Visual associative learning in wood ants. *Journal of Experimental Biology*, **221(3)**, 173260.

Frasnelli, E. (2013). Brain and behavioral lateralization in invertebrates. *Frontiers in psychology*, **4**, 939.

Frasnelli, E., Iakovlev, I., & Reznikova, Z. (2012). Asymmetry in antennal contacts during trophallaxis in ants. *Behavioural brain research*, **232**, 7-12.

Frasnelli, E., Vallortigara, G., Rogers, L.J. (2010). Response competition associated with right-left antennal asymmetries of new and old olfactory memory traces in honeybees. *Behavioural Brian Res.*, **209**, 36-41.

Frasnelli, E., Vallortigara, G. and Rogers, L.J. (2011). Origins of brain asymmetry: Lateralization of odour memory recall in primitive Australian stingless bees. *Behavioural brain research*, **224**, 121-127.

Gazzaniga, M. S. (2000). Cerebral specialization and interhemispheric communication: Does the corpus callosum enable the human condition?. *Brain*, **123**, 1293-1326.

Guerrieri, F. J. and d'Ettorre, P. (2010). Associative learning in ants: conditioning of the maxilla-labium extension response in *Camponotus aethiops*. *Journal of Insect Physiology,* **56**, 88-92.

Guerrieri, F. J., d'Ettorre, P., Devaud, J. M. and Giurfa, M. (2011). Long-term olfactory memories are stabilised via protein synthesis in *Camponotus fellah* ants. *Journal of Experimental Biology*, **214**, 3300-3304.

Guo, Y., Wang, Z., Li, Y., Wei, G., Yuan, J., Sun, Y., Wang, H., Qin, Q., Zeng, Z., Zhang, S. and Chen, R. (2016). Lateralization of gene expression in the honeybee brain during olfactory learning. *Scientific reports*, ***6***, 34727.

Haase, A., Rigosi, E., Frasnelli, E., Trona, F., Tessarolo, F., Vinegoni, C., Anfora, G., Vallortigara, G. and Antolini, R. (2011). A multimodal approach for tracing lateralisation along the olfactory pathway in the honeybee through electrophysiological recordings, morpho-functional imaging, and behavioural studies. *European Biophysics Journal*, **40**, 1247-1258.

Hassall, M., Turner, J., & Girling, R. (2007). Do turning biases by the 7-spot ladybird*, Coccinella septempunctata*, increase their foraging efficiency?. *Behaviour*, **144**, 143-163.

Hori, S., Takeuchi, H. & Kubo, T. (2007). Associative learning and discrimination of motion cues in the harnessed honeybee *Apis mellifera* L. *Journal of Comparative Physiology A,* **193**, 825-833.

Jasmin, J. N., & Devaux, C. (2015). Laterality of leaf cutting in the attine ant *Acromyrmex echinatior*. *Insectes sociaux*, **62**, 109-114.

Jordan, J. T., & Pytte, C. L. (2017). Interhemispheric specialization in the rodent hippocampus: Implications for storage and retrieval of short-and long-term memories. *bioRxiv*, 150193.

Kamimura, Y. (2006). Right-handed penises of the earwig *Labidura riparia* (Insecta, Dermaptera, Labiduridae): evolutionary relationships between structural and behavioral asymmetries. *Journal of morphology*, **267**, 1381-1389.

Kamimura, Y., Mitsumoto, H., & Lee, C. Y. (2014). Duplicated female receptacle organs for traumatic insemination in the tropical bed bug *Cimex hemipterus*: adaptive variation or malformation?. *PloS one*, **9**, e89265.

Kells, A. R., & Goulson, D. (2001). Evidence for handedness in bumblebees. *Journal of Insect Behavior*, **14**, 47-55.

Kight, S. L., Steelman, L., Coffey, G., Lucente, J., & Castillo, M. (2008). Evidence of population-level lateralized behaviour in giant water bugs, *Belostoma flumineum* Say (Heteroptera: Belostomatidae): T-maze turning is left biased. *Behavioural processes*, **79**, 66-69.

Komischke, B., Sandoz, J.C., Malun, D. and Giurfa, M. (2005). Partial unilateral lesions of the mushroom bodies affect olfactory learning in honeybees *Apis mellifera* L. *European Journal of Neuroscience*, **21**, 477-485.

Lang, M., & Orgogozo, V. (2012). Distinct copulation positions in *Drosophila pachea* males with symmetric or asymmetric external genitalia. *Contributions to Zoology*, **81**, 87-94.

Letzkus, P., Ribi, W.A., Wood, J.T., Zhu, H., Zhang, S.W. & Srinivasan, M.V. (2006). Lateralization of olfaction in the honeybee *Apis mellifera*. *Current Biology*, **16**, 1471-1476.

Letzkus, P., Boeddeker, N., Wood, J.T., Zhang, S.W. and Srinivasan, M.V. (2007). Lateralization of visual learning in the honeybee. *Biology letters*, **4**, 16-19.

Malun, D., Giurfa, M., Galizia, C.G., Plath, N., Brandt, R., Gerber, B. and Eisermann, B. (2002). Hydroxyurea-induced partial mushroom body ablation does not affect acquisition and retention of olfactory differential conditioning in honeybees. *Journal of neurobiology*, **53**, 343-360.

Marra, V., O'Shea, M., Benjamin, P. R., & Kemenes, I. (2013). Susceptibility of memory consolidation during lapses in recall. *Nature communications*, **4**, 1578.

Matsuo, R., Kawaguchi, E., Yamagishi, M., Amano, T., & Ito, E. (2010). Unilateral memory storage in the procerebrum of the terrestrial slug *Limax*. *Neurobiology of learning and memory*, **93**, 337-342.

Miler, K., Kuszewska, K., & Woyciechowski, M. (2017). Larval antlions with more pronounced behavioural asymmetry show enhanced cognitive skills. *Biology letters*, **13**, 20160786.

Moorman, S., & Nicol, A. U. (2015). Memory-related brain lateralisation in birds and humans. *Neuroscience & Biobehavioral Reviews*, **50**, 86-102.

Niggebrügge, C., Leboulle, G., Menzel, R., Komischke, B. and de Ibarra, N. H. (2009). Fast learning but coarse discrimination of colours in restrained honeybees. *Journal of Experimental Biology,* **212**, 1344-1350.

Niven, J.E., Bell, A.T.A. (2018). Lessons in Lateralisation from the Insects. *Trends Ecol. Evol*. **33**, 486-488. doi: 10.1016/j.tree.2018.04.008.

Niven, J. E., & Frasnelli, E. (2018). Insights into the evolution of lateralization from the insects. In *Progress in brain research*, **238**, 3-31. Elsevier.

Niven, J. E., & Laughlin, S. B. (2008). Energy limitation as a selective pressure on the evolution of sensory systems. *Journal of Experimental Biology*, **211**, 1792-1804.

Pascual, A., Huang, K. L., Neveu, J., & Préat, T. (2004). Neuroanatomy: brain asymmetry and long-term memory. *Nature*, **427**, 605.

Pavlov, I. (1897). *The work on digestive glands (WH Tromso Trans)*. London: Griffin.

Peters, R. S., Krogmann, L., Mayer, C., Donath, A., Gunkel, S., Meusemann, K., Kozlov, A., Podsiadlowski, L., Petersen, M., Lanfear, R. & Diez, P. A. (2017). Evolutionary history of the Hymenoptera. *Current Biology*, **27**, 1013-1018.

Rigosi, E., Frasnelli, E., Vinegoni, C., Antolini, R., Anfora, G., Vallortigara, G. and Haase, A. (2011). Searching for anatomical correlates of olfactory lateralization in the honeybee antennal lobes: a morphological and behavioural study. *Behavioural brain research*, ***221***, 290-294.

Rogers, L. J. (2014). Asymmetry of brain and behavior in animals: Its development, function, and human relevance. *Genesis*, **52**, 555-571.

Rogers, L. J., & Andrew, R. (Eds.). (2002). *Comparative vertebrate lateralization*. Cambridge University Press.

Rogers, L.J. and Vallortigara, G. (2008). From antenna to antenna: lateral shift of olfactory memory recall by honeybees. *PLoS One*, ***3***, 2340.

Rogers, L. J., Rigosi, E., Frasnelli, E., & Vallortigara, G. (2013). A right antenna for social behaviour in honeybees. *Scientific reports*, **3**, 2045.

Rogers, L.J., Vallortigara, G. (2019). Complementary specialisations of the left and right sides of the honeybee brain. *Front. Psychol.*, **10**, 280.

Romano, D., Donati, E., Canale, A., Messing, R. H., Benelli, G., & Stefanini, C. (2016). Lateralized courtship in a parasitic wasp. *Laterality: Asymmetries of Body, Brain and Cognition*, **21**, 243-254.

Romano, D., Kavallieratos, N. G., Athanassiou, C. G., Stefanini, C., Canale, A., & Benelli, G. (2016). Impact of geographical origin and rearing medium on mating success and lateralization in the rice weevil, *Sitophilus oryzae* (L.)(Coleoptera: Curculionidae). *Journal of Stored Products Research*, **69**, 106-112.

Sandoz, J.C., Galizia, C.G. and Menzel, R. (2003). Side-specific olfactory conditioning leads to more specific odor representation between sides but not within sides in the honeybee antennal lobes. *Neuroscience*, **120**, 1137-1148.

Sandoz, J.C. and Menzel, R. (2001). Side-specificity of olfactory learning in the honeybee: generalization between odors and sides. *Learning & Memory*, **8**, 286-294.

Simões, P., Ott, S. R. and Niven, J. E. (2011). Associative olfactory learning in the desert locust, *Schistocerca gregaria*. *Journal of Experimental Biology,* **214**, 2495-2503.

Figures

Figure 1. Ants subjected to a paired training with unilateral and bilateral reinforcement of antennal touch with sugar learn the association between a visual cue and a sucrose reward. A) The performance of ants subjected to any type of paired training increases significantly during training. Responses of paired ants with reinforcement to the right antenna (PR, dark red; N=88) or the left (PL, dark blue; N=92) show no significant difference, but are both significantly lower than paired ants with reinforcement to both antennae (PB, dark gray; N= 62). B) Ants subjected to unpaired training with reinforcement to the right (UPR, light red; N=83) or left antenna (UPL, blue; N=89) respond significantly less than paired trained ants. Ants that see the CS but do not touch the sugar reward with their antennae or their mouthparts (CSO, gray; N=81) show no increase in MaLER performance with training trial. Likewise, ants that see the CS but do not touch the sugar reward with their antennae and then are able to feed (NTA, purple; N=59) show no increase in MaLER performance with training trial.

Figure 2. Wood ants retain visual associative memories and recall those memories either 10 minutes and 1 hour or 24 hours after training, depending on which antenna was reinforced during training. A) Percentage of MaLER performance to the CS at 10 minutes. PR (dark red; N=37) ants are not significantly different than PB ants (dark gray; N=15), but are significantly higher (*) than UPR (light red; N=36) and PL (dark blue; N=37). PL ants do not perform MaLER significantly more than UPL ants (light blue; N=38). B) Percentage of MaLER performance to the CS at 1 hour. PR (N=51) ants are not significantly different from PB ants (N=47), but are significantly higher than UPR (*; N=47) and PL (*; N=55). PL ants do not perform MaLER significantly more than UPL ants (N=51). C) Percentage of MaLER performance to the CS at 24 hours. PL (N=40) ants' responses are significantly higher than PR (*; N=40) but not PB (N=22) nor UPL ants (N=41). PR ants and indistinguishable from UPR (N=38). A-C) NTA (10 min, N=20; 1h, N=39; 24h, N=36) and CSO (10 min, N=19; 1h, N=62; 24h, N=47) are represented in purple and light gray, respectively.